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Molecular Phylogeny of *Aristolochia* (Aristolochiaceae) Inferred from *matK* Sequences

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A molecular phylogenetic analysis of *Aristolochia* subgenera *Siphisia* and *Aristolochia* (Aristolochiaceae) was conducted based on the nucleotide sequence variation in the *matK* gene. Sixty seven samples covering much of the taxonomic/morphological diversity within the two subgenera, excepting subseries *Anthocaulicae*, were analyzed using *Saruma henryi*, *Asarum yakusimense* and *Thottea tomentosa* as outgroups. The phylogenetic tree obtained was generally consistent with the current classification system and discrepancies were found only in a small portion concerning subsect. *Pentandrae* and its allies. The species of subgenus *Siphisia* from Japan and Taiwan and *A. mollissima* from southern China form a terminal monophyletic clade with few site changes found among them.

Key words: *Aristolochia*, Aristolochiaceae, *matK*, phylogeny, systematics

The genus *Aristolochia* (Aristolochiaceae) has been treated as a single large genus (Duchartre 1864, Schmidt 1935, Shaw 1973, Ma 1989, Gonzalez 1999), while Rafinesque (1828), Klotzch (1859) and Huber (1960, 1993) recognized some small genera corresponding to infrageneric taxa recognized by others. At present, 350 - 430 species, mostly distributed in tropical and subtropical regions, are recognized. Many of the species are woody lianas, rhizomatous perennial herbs with twining stems or rarely subshrubs. They are commonly characterized by a long tubular perianth, which is enlarged at the base (called a utricle) and encloses the gynostemium. The systematics of *Aristolochia* has been based on morphological criteria, such as the shape and hairiness of the leaves, the shape of the perianth tube, the number of styles and anthers on the gynostemium and the capsule dehiscence. It has been generally accepted that the group with indehiscent fruits and evenly trilobed, straight perianth tube (*Aristolochia* subgenus *Pararistolochia*) is more primitive than the other two groups with dehiscent fruits and curved perianth tube (subgenera *Aristolochia* and *Siphisia*), but no phylogenetic analysis of any level has been undertaken. Our objectives were to infer phylogenetic relationships within the two putative advanced subgenera, *Aristolochia* and *Siphisia*, using molecular data and to compare the implications of our results on the systematics of *Aristolochia* with those of Gonzalez (1999) (Table 1) and others. Our sample covered much of the taxonomic/morphological diversity within the two subgenera excepting subseries *Anthocaulicae*. Because preliminary examination of the *matK* gene sequence was found to give good resolution of infrageneric relationships, we used it in our analysis.

TABLE 1. Infrageneric classification system proposed by Gonzalez (1999). Nomenclature is altered according to International Code of Botanical Nomenclature (Saint Louis Code).

- *Aristolochia* L.
  - subgen. *Siphisia* (Rafin.) Duchartre
  - subgen. *Aristolochia*
    - sect. *Aristolochia*
      - subsect. *Aristolochia*
      - subsect. *Podanthemum* (Klotzsch) Schmidt
    - sect. *Gymnolobus* Duchartre
      - subsect. *Pentandrae* Duchartre
      - subsect. *Hexandrae* Duchartre
        - ser. *Thyrsicae* F. Gonzalez
        - ser. *Hexandrae* F. Gonzalez
          - subser. *Anthocaulicae* F.Gonzalez
          - subser. *Hexandrae* F. Gonzalez
  - subgen. *Pararistolochia* Hutch. et Dalz.

Materials and Methods

Plant materials were collected from natural populations or cultivated plants. Most of the plants obtained from natural populations were grown in the Medicinal Botanical Garden, Faculty of Pharmaceutical Sciences, Setsunan University. Flowers were used to identify the samples. Sixty seven samples covering much of the taxonomic/morphological diversity within *Aristolochia* subgenera *Aristolochia* and *Siphisia* were selected; samples of subseries *Anthocaulicae* were not available. Three species, *Saruma henryi*, *Thottea tomentosa* and *Asarum yakusimense*, were selected as outgroups based on the results of Qiu *et al.* (2000) (Table 2). Voucher specimens are deposited in the Herbarium of the University of Tokyo (TI).

Dry or fresh leaves were frozen using liquid nitrogen and pulverized to a fine powder. Before DNA extraction, the leaf powder was suspended in HEPES buffer (pH 8.0) and centrifuged at 12,500 rpm at 20°C for 5 min to remove sticky polysaccharides (Setoguchi & Ohba 1995). Total DNA was isolated from the pellet using the CTAB method of Hasebe & Iwatsuki (1990), and purified by CIA (chloroform: Isopropanol=25:1) and PCI (CIA: Phenol=1:1). The double-stranded DNA of a part of the *matK* gene of cpDNA (ca. 1,250bp) was amplified by 40 cycles of symmetric polymerase chain reaction (PCR) using primers AF and 8R of Ooi *et al.* (1995) for the *matK* gene. PCR cycle conditions in the first cycle consisted of 1.5 min at 96°C for denaturation, 1 min at 55°C for primer annealing, and 2 min at 72°C for primer extension. In the last cycle, the time at 72°C was increased to 10 min. PCR products were purified by electrophoresis in 0.5% agarose gel using 1 x TAE buffer. The gel was stained with ethidium bromide and the DNA was eluted using Geneclean II (Bio 101, Vista, California, USA). Purified DNA were sequenced in both directions by the standard methods of the Big dye terminator cycle sequencing kit (Perkin Elmer, Foster City, California, USA) on an Applied Biosystems Model 377 automated sequencer (Applied Biosystems, Foster City, California, USA). Sequence Primers used AF, 8R, 1412F and 1470R of Ooi *et al.* (1994), and Johnson & Soltis (1994). Sequence data were aligned with the CLUSTAL X program (Thompson 1997). Insertions and deletions were excluded from the sequence matrix.

TABLE 2. Species of Aristolochiaceae sequenced for *matK*

Species	**GenBank accession No.**	**collection data**	**Voucher**
Saruma henryi Oliver	AB060736	China (cult)	SETS36
Asarum yakusimense Masamune	AB060737	Yakushima, Japan (cult2)	J. Murata, s.n.
Thottea tomentosa (Bl.) Ding Hou	AB060738	W. Java (cult2)	J.Murata 01-2
Aristolochia subgen. *Siphisia*			
A. arborea Linden	AB060739	cult. in Missouri Bot. Gard.	J. Murata 99-1
A. austroyunnanensis S.M. Hwang	AB060740	Guangxi, China (cult)	SETS15
A. cucurbitifolia Hayata	AB060741	Taiwan (cult)	SETS39
A. kaempferi Willd.	AB060743	Shizuoka, Japan (cult)	SETS12
A. kwangsiensis C.F. Liang	AB060744	Guangxi, China (cult)	SETS28
A. liukiuensis Hatus.	AB060745	Okinawa, Japan (cult)	SETS26
A. liukiuensis Hatus.	AB060746	Okinawa, Japan (cult)	SETS40
A. liukiuensis Hatus.	AB060747	Okinawa, Japan (cult)	SETS34
A. macrophylla Lam.	AB060742	U.S.A (cult)	SETS3
A. manshuriensis Kom.	AB060748	S. Korea (cult)	SETS21
A. mollissima Hance	AB060749	China (cult)	SETS30
A. moupinensis Franch.	AB060750	Sichuan, China (cult)	SETS52
A. moupinensis Franch.	AB060751	Yunnan, China (cult)	SETS9
A. nakaoi F. Maekawa	AB060752	Nepal (cult)	SETS14
A. onoei Franch. & Sav.	AB060753	Amami-oshima, Japan (cult)	SETS43
A. onoei Franch. & Sav.	AB060754	Iriomote Is., Japan (cult)	SETS41
A. onoei Franch. & Sav.	AB060755	Amami-oshima, Japan (cult)	SETS44
A. onoei Franch. & Sav.	AB060756	Hyogo, Japan (cult)	SETS7
A. onoei Franch. & Sav.	AB060757	Amami-oshima, Japan (cult)	SETS18
A. paracleta Pfeifer	AB060758	Mt. Uyuca, Honduras (cult)	J. Murata et al. 97-206
A. salvadorensis Standl.	AB060759	Guatemala (cult)	J.Murata 00-3
A. shimadai Hayata	AB060760	Taichung, Taiwan (cult)	SETS38
A. shimadai Hayata	AB060761	Changhua, Taiwan (cult)	SETS50
A. shimadai Hayata	AB060762	Kenting, Taiwan (cult)	SETS51
A. shimadai Hayata	AB060763	Taiwan (cult)	SETS65
A. tricaudata Lem.	AB060764	Mexico (cult)	SETS42
A. westlandii Hemsl.	AB060765	China (cult)	SETS36
subgen. *Aristolochia*			
sect. *Aristolochia*			
subsect. *Aristolochia*			
A. baetica L.	AB060766	Spain (cult)	SETS5
A. clematitis L.	AB060767	Europe (cult)	SETS17
A. debilis Siebold & Zucc.	AB060768	Tokyo, Japan (cult)	SETS10
A. foveolata Merr.	AB060769	Taiwan (cult)	SETS4
A. goliathiana M.J. Parsons	AB060770	South Kalimantan (cult)	SETS19
A. papilliifolia Ding Hou	AB060772	East Kalimantan (cult2)	J. Murata 00-4
A. sp. JM01 (subsect. *Aristolochia*)	AB060773	Guangxi, China (cult)	SETS16
A. sp. JM02 (subsect. *Aristolochia*)	AB060774	Assam, India (cult)	SETS49
subsect. *Podanthemum*			
A. gaudichaudii Duchtr.	AB060775	Indonesia (cult)	SETS8
A. indica L.	AB060771	Mt. Popa, Myanmar (cult)	J. Murata 00-2
A. jackii Steud.	AB060776	Madagascar (cult)	SETS56
A. kankauensis Sasaki	AB060777	Taiwan (cult)	SETS35
A. pierrei Lecomte	AB060778	Thailand (cult)	SETS64
A. tagala Cham.	AB060779	New Guinea (cult)	SETS27
A. tagala Cham.	AB060780	Nirgili, India (cult)	SETS48

sect. *Gymnolobus*			
subsect. *Pentandrae*			
A. nelsonii Eastwood	AB060781	Chiapas, Mexico	J. Murata et al. 98-212
subsect. *Hexandrae*			
ser. *Thyrsicae*			
A. maxima L.	AB060782	Costa Rica (cult3)	J. Murata 01-1
A. maxima L.	AB060783	S. America (cult)	SETS37
A. maxima L.	AB060784	Chiapas, Mexico	J. Murata et al. 98-208
A. ovalifolia Duchtr.	AB060785	Mt. El. Chile, Honduras (cult)	SETS66
ser. *Hexandrae*			
A. arcuata Mast.	AB060786	(cult)	SETS29
A. argentina Griseb.	AB060787	(cult)	SETS33
A. burelae Herzog	AB060788	Bolivia (cult5)	J. Murata 00-1
A. cymbifera var. *abbreviata* Duchtr.	AB060789	(cult)	SETS55
A. elegans Mast.	AB060790	(cult)	SETS24
A. fimbriata Cham. & Schltdl.	AB060791	S. America (cult)	SETS11
A. giberti Hook.	AB060792	Paraguay (cult)	SETS2
A. gigantea Hook.	AB060793	(cult)	SETS22
A. gigantea Hook.	AB060794	(cult4)	SETS67
A. macroura Duchtr.	AB060795	(cult)	SETS23
A. macroura Duchtr.	AB060796	(cult4)	SETS69
A. maurorum L.	AB060797	(cult3)	SETS54
A. odoratissima L.	AB060798	(cult)	SETS31
A. peruviana O.C. Schmidt	AB060799	(cult4)	SETS70
A. ringens Vahl	AB060800	(cult)	SETS57
A. ringens Vahl	AB060801	(cult)	SETS13
A. pilosa Kunth	AB060802	Ceiba, Honduras (cult)	SETS6
A. triangularis Cham.	AB060803	(cult)	SETS20
A. trilobata L.	AB060804	(cult)	SETS1
A. trilobata L.	AB060805	(cult4)	SETS68

cult – cultivated in Medicinal Plant Garden, Setsunan Univ.
cult2 – cultivated in Bot. Gard. Univ. Tokyo
cult3 – cultivated in Bot. Gard. Osaka City Univ.
cult4 – cultivated in Kyoto Bot. Gard.
cult5 – cultivated in Makino Bot. Gard.

The phylogenetic analyses were performed with PAUP* (version 4.0; Swofford 2001) using maximum parsimony and neighbor-joining. The maximum parsimony (MP) analysis was conducted using the heuristic search option with 100 replicates of random addition, Tree Bisection Reconnection (TBR) branch-swapping algorithm, and MULTREES on, and all characters were equally weighted in the analyses. The neighbor-joining (NJ) tree was constructed using the Kimura's two-parameter distance (Kimura 1980). The branch supports were assessed by bootstrap analyses (Felsenstein 1985) with 1,000 bootstrap replicates. For parsimony analysis, the heuristic search option with 100 replicates of random addition, TBR branch-swapping, and MULTREES off.

Results

Sequences of the *matK* gene for 70 samples were obtained and deposited in DDBJ (see Table 2 for accession numbers). The length of the *matK* gene sequences ranged from 1,194 to 1,224 bp among the genus *Aristolochia*. The aligned length among all accessions was 1,179

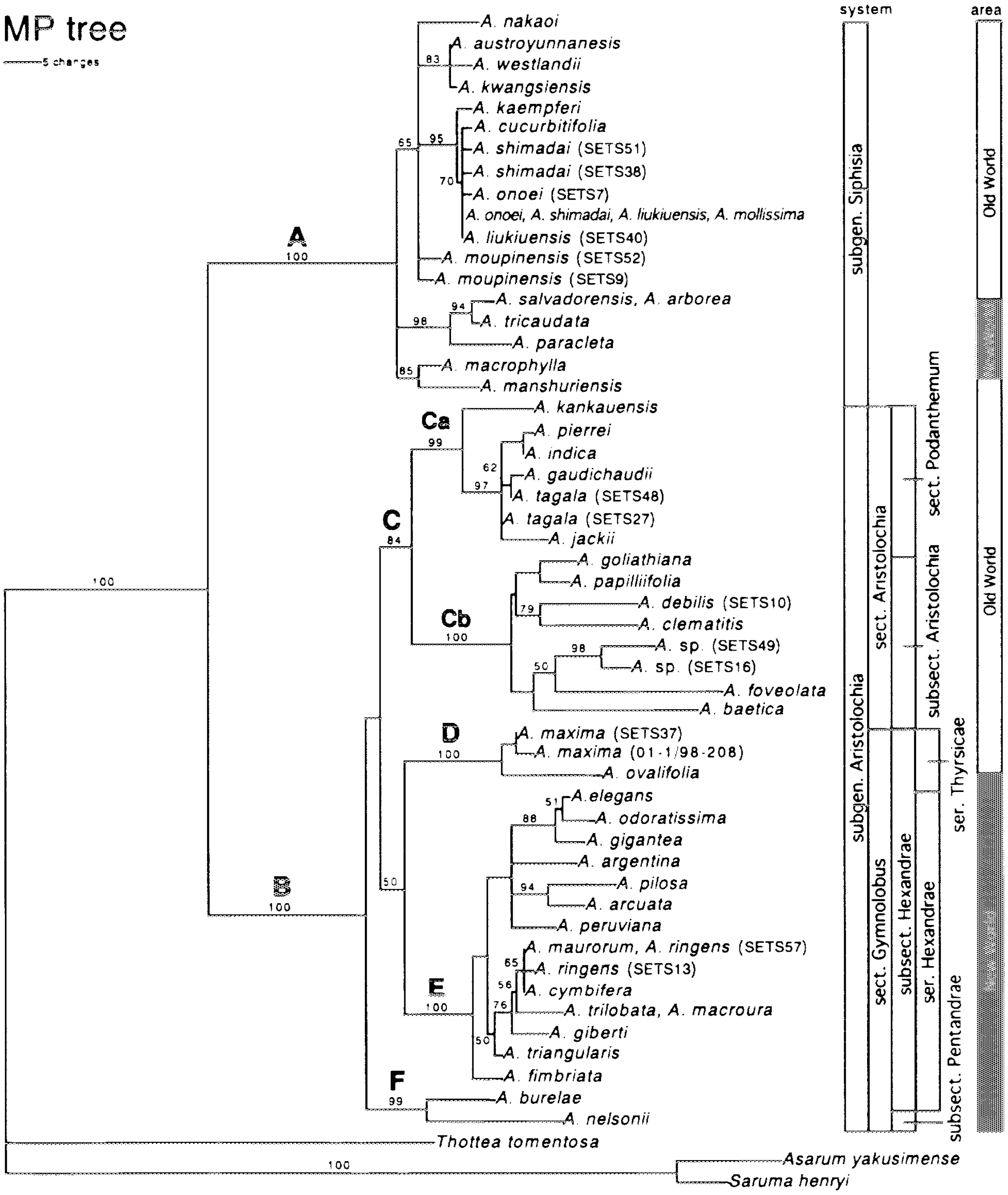

FIG. 1. Strict consensus tree of 368 equally parsimonious ones for the genus *Aristolochia* based on *matK* gene sequences. Length = 625 steps, CI = 0.712, RI = 0.899, RC = 0.640. A percentage of the 1,000 bootstrap values (>50%) is given for each node. Alphabets (A, B, C, Ca, Cb, D, E, F) show the major clades.

bp, excluding insertions and deletions. It contained 366 variable sites, of which 246 sites were phylogenetically informative.

The MP analysis resulted in 368 most parsimonious trees of 625 steps with a Consistency Index (CI) of 0.712, a Retention Index (RI) of 0.899 and a Rescaled Consistency Index (RC) of 0.640 under the equal weighting criterion. The strict consensus tree of the parsimonious trees is shown in Fig. 1 with bootstrap values. The topology of the neighbor-joining tree (Fig. 2) is similar to the MP tree (Fig. 1): major clades are nearly consistent between the two trees, excepting the position of

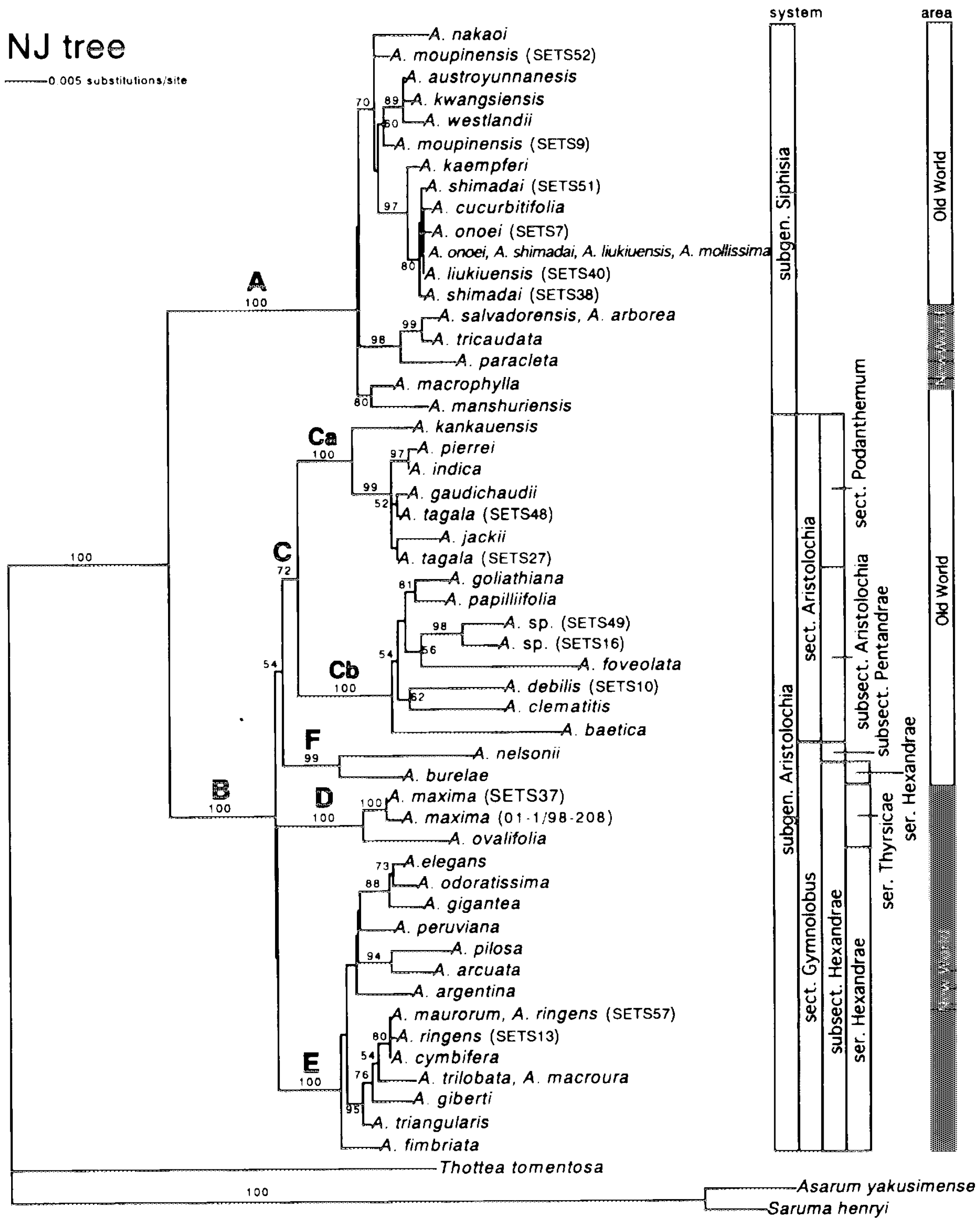

FIG. 2. Neighbor-joining tree for the genus *Aristolochia* based on *matK* gene sequences using Kimura's two-parameter distance. A percentage of the 1000 bootstrap values (>50%) is given for each node. Letters (A, B, C, Ca, Cb, D, E, F) show the major clades.

the clade with *A. nelsonii* and *A. burelae* (F). The monophyly of the genus *Aristolochia* (although subgenus *Pararistolochia* was not examined) was highly supported in both MP and NJ trees with bootstrap values 100%, respectively. Major subclades in the genus generally have good bootstrap support. Intraspecific variation was found in *A. liukiuensis*, *A. maxima*, *A. moupinensis*, *A. onoei*, *A. ringens*, *A. shimadai* and *A. tagala*.

Discussion

In discussing the phylogenetic implications of our results we mostly consider Gonzalez's (1999) system (Table 1), which well reflexes previous treatments by different authors. Gonzalez's system and the phylogenetic tree (Fig. 1) were generally consistent. Discrepancies were found only in a small portion concerning subsect. *Pentandrae* and its allies.

Subgen. *Siphisia* is morphologically well defined by having basally dehiscent capsules, a trilobed perianth tube and a gynostemium with three lobes each accompanying two anthers. In contrast, subgen. *Aristolochia* has apically dehiscent capsules, a bilobed or undivided perianth tube and a gynostemium with five or more than six lobes each accompanying a single anther. In the tree obtained, monophyly of subgenera *Siphisia* and *Aristolochia* was supported with bootstrap values of 100% (in both clade A and B).

In clade A, three subclades were recognized. The first comprised three species of subshrub and one liana from Central America. The second consisted of *A. manshuriensis* of eastern Asia and *A. macrophylla* of eastern North America. The third included all species from Asia, excepting *A. manshuriensis*. In the third clade, all species of subgen. *Siphisia* from Japan and Taiwan and *A. mollissima* from southen China formed a clade supported by a bootstrap value 96%, showing very close relationships (only 0 - 1 site changes between species in examined *matK*), although some species are quite distinct morphologically.

Subgen. *Aristolochia* is subdivided into two sections *Aristolochia* and *Gymnolobus*. Sect. *Aristolochia* is defined as an Old World group characterized morphologically by the collar-like expansion of the gynostemium lobes covering the anthers before anthesis. Sect. *Gymnolobus* is defined as a New World group which lacks the collar-like expansion on the gynostemiun. In our tree, the species of section *Aristolochia* were all included in a single clade (clade C), which was supported by a relatively high bootstrap value (84% in MP tree and 72% in NJ tree). Clade C was subdivided into two distinct clades Ca and Cb, which correspond to subsections *Podanthemum* and *Aristolochia*, respectively. Morphologically subsect. *Podanthemum* is distinguished from subsect. *Aristolochia* by the unique stipitate utricle of the perianth tube. Clade E consists exclusively of species from sect. *Gymnolobus* ser. *Hexandrae*.

Species of sect. *Gymnolobus* also occur in two small clades outside of clade E: One (clade D) consists of *A. maxima* and *A. ovalifolia* and the other (clade F) consists of *A. nelsonii* and *A. burelae*. Clade D is correspondent to ser. *Thyrsicae*, which was recently recognized by Gonzalez (1991) based on the thyrsoid branching in the inflorescence. It is remarkable that clade F is connected to Clade C of sect. *Aristolochia* in the NJ tree, although the bootstrap value is not high (54%). If the NJ tree reflects true phylogeny, subsect. *Hexandrae* is not monophyletic and sectional delimitation between sects. *Aristolochia* and *Gymnolobus* should be reconsidered. *Aristolochia nelsonii*, endemic to Mexico and Central America, belongs to subsect. *Pentandrae*, which is characterized by having a unique pentamerous gynostemium. The tree obtained also suggests that subsect. *Pentandrae* evolved from an ancestor with a hexamerous gynostemium such as *A. burelae*.

The evolution of chromosome numbers in *Aristolochia* is a very interesting topic and differentiation of the base number between subgenera *Siphisia* and *Aristolochia* has been suggested (Ma 1989). At present, however, more chromosomal data is needed to understand the significance for the systematics of the genus.

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